PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 11 April 2001 (11.04.01)	
International application No. PCT/KR00/00810	**Applicant's or agent's file reference** AP-054
International filing date (day/month/year) 26 July 2000 (26.07.00)	**Priority date** (day/month/year) 27 July 1999 (27.07.99)
Applicant LEE, Hyune, Hwan et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

27 February 2001 (27.02.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference AP-054	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. **PCT/KR00/00810**	International filing date *(day/month/year)* 26 JULY 2000 (26.07.2000)	Priority date *(day/month/year)* 27 JULY 1999 (27.07.1999)

International Patent Classification (IPC) or national classification and IPC

IPC7 C12N 15/12

Applicant

LEE, HYUNE HWAN et al

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   [X] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [ ] Certain observations on the international application

Date of submission of the demamd	Date of completion of this report
27 FEBRUARY 2001 (27.02.2001)	22 DECEMBER 2001 (22.12.2001)

Name and mailing address of the IPEA/KR	Authorized officer
Korean Intellectual Property Office Government Complex-Daejeon, Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea	AHN, Mi Jung
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5593

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International aplication No.
PCT/KR00/00810

I. Basis of the report

1. With regard to the elements of the international application:*

☐ the international application as originally filed

☒ the description:
pages 1-3, 5-12 , as originally filed
pages , filed with the demand
pages 4, 4A , filed with the letter of Feb 27, 2001

☒ the claims:
pages 13, 14 , as originally filed
pages , as amended (together with any statment) under Article 19
pages , filed with the demand
pages , filed with the letter of

☒ the drawings:
pages 1/4 - 4/4 , as originally filed
pages , filed with the demand
pages , filed with the letter of

☐ the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , filed with the letter of

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application(under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained inthe international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disc losure in the international applicationas as filed has been furinshed.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages 4

☐ the claims, Nos.

☐ the drawings, sheet

5. ☐ This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box(Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed." and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.*

V. **Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1, 2, 4-6	YES
	Claims	3	NO
Inventive step (IS)	Claims	1, 2, 4-6	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-6	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

D : Salmon et al (Protein Expr Purif. vol.9 : 203, 1997)

I. Novelty and Inventive step

The present invention defined by claims relates to a recombinant human lactoferrin produced by using an insect cell, Sf9, and to a method producing the same. Additionally, it includes a method to verify the biological activity of a recombinant lactoferrin by measuring extinct rate of disease-causing germs through the mixing experiment.

Previously, Salmon et al (1997) have demonstrated the expression of human lactoferrin in Sf9 cells using baculovirus. Compared with the prior art, the present invention not only adopts the more stepwised method to produce recombinant lactoferrin but also shows the more efficient production system (250mg/l vs. 15mg/l). In addition, the verification method of biological activity in the present invention has not been disclosed in the prior arts. Therefore, claim 1, 2, 4-6 seem to satisfy the requirement of Article 33(2) and (3) of PCT

However, human lactoferrin presented in claim 3 is considered to be the same material disclosed in the work of Salmon et al. Moreover, human lactoferrin itself is the well-known material as described in Specification of the present invention (p1, line 14). Therefore, claim 3 fails to meet the requirement of Article 33(2) of PCT.

II. Industrial Applicability

The subject matter of claims 1-6 meets the criteria set out in Article 33(4) of PCT.

It is also of great importance to verify the biological activity of the produced recombinant *lactoferrin*, since the normal bacteria or yeasts are mostly improper as a host due to the anti-bacterial activity of *lactoferrin* itself and, if used as a host, inadequate to industrial uses in such a small quantity available. To solve this problem, Ward et al. (1992) successfully produced the recombinant *lactoferrin* using fungi such as *Aspergillus indulans* or *oryzae*. But, there was a limitation in that the *lactoferrin* was expressed in such a small amount of 5 to 25 mg/l in relation to the *lactoferrin* expressed from an insect cell. Furthermore, because the recombinant *lactoferrin* produced from the fungi was analyzed for its biological activity only in terms of affinity with Fe^{58} labeled with radioactive isotopes, there was still remained a question as to whether the recombinant *lactoferrin* actually had an anti-bacterial action on the pathogenic microorganisms.

Meanwhile, studies concerning production of human *lactoferrin* using cell of the higher animals were attempted, but because of the high cost concerning the cultivation process, it is said that the application is difficult.

Thus, researchers in this art have shown some interest in producing human *lactoferrin* using insects cell which can be massively produced at a low cost. For example, it was reported that there were studies done in producing human *lactoferrin* using insects cell(Salmon et al., "Characterization of Human Lactoferrin Produced in the Baculovirus Expression System" in Protein Expr. Purif., vol. 9(2), 203-210, 1997). However, with such method, obtained human *lactoferrin* were very small amount of 15 mg/l. It was estimated that because of the sequence of human *lactoferrin* secretion did not well worked on insect cell. Also, 5% calf serum was used in such method, as a result, purification of human *lactoferrin* was bad.

To overcome the above problems, the present inventor has contrived a novel method for mass-production of human *lactoferrin* in a simple way and a

It is also of great importance to verify the biological activity of the produced recombinant *lactoferrin*, since the normal bacteria or yeasts are mostly improper as a host due to the anti-bacterial activity of *lactoferrin* itself and, if used as a host, inadequate to industrial uses in such a small quantity available. To solve this problem, Ward et al. (1992) successfully produced the recombinant *lactoferrin* using fungi such as *Aspergillus indulans* or *oryzae*. But, there was a limitation in that the *lactoferrin* was expressed in such a small amount of 5 to 25 mg/l in relation to the *lactoferrin* expressed from an insect cell. Furthermore, because the recombinant *lactoferrin* produced from the fungi was analyzed for its biological activity only in terms of affinity with Fe^{58} labeled with radioactive isotopes, there was still remained a question as to whether the recombinant *lactoferrin* actually had an anti-bacterial action on the pathogenic microorganisms.

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To overcome the above problems, the present inventor has contrived a novel

method for mass-production of human *lactoferrin* in a simple way and a verification method for the biological activity of the recombinant human *lactoferrin*.

It is, therefore, an object of the present invention to provide a method for massively producing human *lactoferrin* using an insect cell.

It is another object of the present invention to provide a recombinant insect cell for producing a human *lactoferrin* protein.

It is still another object of the present invention to provide a biological verification method for a recombinant *lactoferrin* produced from an insect cell.

DISCLOSURE OF INVENTION

The present invention relates to a method for producing human *lactoferrin* comprising the steps of transducing a human lactoferrin gene into an insect cell by gene recombination, cloning and expressing human lactoferrin in the insect cell, and using the insect cell to produce the human lactoferrin. The present invention also relates to a

AMENDED SHEET(ART. 34)

PA NT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference AP-054	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/KR00/00810	International filing date *(day/month/year)* 26 JULY 2000 (26.07.2000)	(Earliest) Priority Date *(day/month/year)* 27 JULY 1999 (27.07.1999)

Applicant

LEE, HYUNE HWAN et al

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
   ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:
   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ the statement that the subsequenlty furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (See Box II).

4. With regard to the title,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawing to be published with the abstract is Figure No. 1
   ☒ as suggested by the applicant.
   ☐ None of the figures.
   ☐ because the applicant failed to suggest a figure.
   ☐ because this figure better characterizes the invention.

INTERNATIONA. EARCH REPORT

ernational application No.
[illegible]/KR00/00810

A. CLASSIFICATION OF [illegible]ECT MATTER

IPC7 C12N 15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

C12N15/12, 15/70, 15/74 ; C07K 14/79, 14/435

Doeumentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Korean Patents and Applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)

IBM, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GILLESPIE LS, HILLESLAND KK and KNAUER DJ 'Expression of biological active human antithrombon III by recombinant baculovirus in Spodopters frugiperda cells' In : J. Biol. Chem., vol. 266 (6), 1991, p.3995-4001 see the abstract	1-6
Y	SALMON V, LEGRAND D, GEORGES B, SLOMIANNY MC, CODDEVILLE B and SPIKE G 'Characterization of human lactoferrin produced in the baculovirus expression system' In : Protein Expr. Purif., vol. 9 (2), 1997, p.203-210 see the whole document	1-6
Y	US 5,766,939 (Baylor College of Medicine) 16 Jun 1998 see the whole document	1-6

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 OCTOBER 2000 (30.10.2000)	31 OCTOBER 2000 (31.10.2000)

Name and mailing address of the ISA/KR
Korean Industrial Property Office
Government Complex-Taejon, Dunsan-dong, So-ku, Taejon
Metropolitan City 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
Mi-Chung Ahn
Telephone No. 82-42-481-5593

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR00/00810

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5,766,939	16. 06. 98	WO 93/22348 A1	11. 11. 93
		US 6100054	08. 08. 00
		US 6080559	27. 06. 00
		US 5955316	21. 09. 99
		US 5849881	15. 09. 88

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REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No. PCT/KR 00/00310

International Filing Date 26 July 2000 (26.07.00)

Name of receiving Office and "PCT International Application": Korean Industrial Property Office P C T International Application

Applicant's or agent's file reference *(if desired) (12 characters maximum)*: AP-054

Box No. I TITLE OF INVENTION A HUMAN LACTOFERRIN PRODUCED BY USING AN INSECT CELL AND METHOD USING THE SAME

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

LEE, HYUNE HWAN
9-802, SAMICK APT, MYUNGIL-DONG 134-780
KANGDONG-GU, SEOUL, REPUBLIC OF KOREA

[V] This person is also inventor.

Telephone No. 82-31-330-4280

Facsimile No. 82-31-333-1696

Teleprinter No.

State *(that is, country)* of nationality: KR

State *(that is, country)* of residence: KR

This person is applicant for the purposes of: [V] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

CHANG, YUN-HEE
978-13 (34/2), GYESAN-1 DONG, GYEYANG-GU,
INCHON-SI, 407-051, REPUBLIC OF KOREA

This person is:

[ ] applicant only

[V] applicant and inventor

[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: KR

State *(that is, country)* of residence: KR

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [V] the United States of America only [ ] the States indicated in the Supplemental Box

[X] Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: [V] agent [ ] common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

KOH, YOUNG-HOE
SEOCHO-GU SEOCHO-DONG 1543-11
SANWOO BLDG. 4TH FLOOR, SEOUL
137-070, REPUBLIC OF KOREA
(E-MAIL:MAIL@PATINFO.COM)

Telephone No. 82-2-584-7777

Facsimile No. 82-2-584-7337

Teleprinter No.

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Sheet No. .2....

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

KWEON, CHANG-HEE
A-202, JANGMI APT, 706-17 KUMJUNG-DONG,
KUNPO-SI, KYUNGGI-DO, 435-050,
REPUBLIC OF KOREA

This person is:
- ☐ applicant only
- ☑ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: KR
State *(that is, country)* of residence: KR

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☑ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:
State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:
State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:
State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☑ AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☑ EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☑ EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☑ OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☑ AE United Arab Emirates
- ☑ AG Antigua and Barbuda
- ☑ AL Albania .....
- ☑ AM Armenia .....
- ☑ AT Austria .....
- ☑ AU Australia .....
- ☑ AZ Azerbaijan
- ☑ BA Bosnia and Herzegovina .....
- ☑ BB Barbados
- ☑ BG Bulgaria .....
- ☑ BR Brazil .....
- ☑ BY Belarus .....
- ☑ BZ Belize
- ☑ CA Canada
- ☑ CH and LI Switzerland and Liechtenstein
- ☑ CN China .....
- ☑ CR Costa Rica .....
- ☑ CU Cuba .....
- ☑ CZ Czech Republic .....
- ☑ DE Germany .....
- ☑ DK Denmark .....
- ☑ DM Dominica
- ☑ DZ Algeria .....
- ☑ EE Estonia .....
- ☑ ES Spain .....
- ☑ FI Finland .....
- ☑ GB United Kingdom
- ☑ GD Grenada
- ☑ GE Georgia .....
- ☑ GH Ghana .....
- ☑ GM Gambia
- ☑ HR Croatia .....
- ☑ HU Hungary .....
- ☑ ID Indonesia
- ☑ IL Israel .....
- ☑ IN India .....
- ☑ IS Iceland
- ☑ JP Japan .....
- ☑ KE Kenya .....
- ☑ KG Kyrgyzstan .....
- ☑ KP Democratic People's Republic of Korea .....
- ☐ KR Republic of Korea .....
- ☑ KZ Kazakhstan .....
- ☑ LC Saint Lucia
- ☑ LK Sri Lanka
- ☑ LR Liberia
- ☑ LS Lesotho .....
- ☑ LT Lithuania
- ☑ LU Luxembourg
- ☑ LV Latvia
- ☑ MA Morocco .....
- ☑ MD Republic of Moldova .....
- ☑ MG Madagascar .....
- ☑ MK The former Yugoslav Republic of Macedonia .....
- ☑ MN Mongolia
- ☑ MW Malawi .....
- ☑ MX Mexico .....
- ☑ MZ Mozambique
- ☑ NO Norway
- ☑ NZ New Zealand .....
- ☑ PL Poland .....
- ☑ PT Portugal .....
- ☑ RO Romania
- ☑ RU Russian Federation .....
- ☑ SD Sudan
- ☑ SE Sweden
- ☑ SG Singapore
- ☑ SI Slovenia .....
- ☑ SK Slovakia .....
- ☑ SL Sierra Leone .....
- ☑ TJ Tajikistan .....
- ☑ TM Turkmenistan .....
- ☑ TR Turkey .....
- ☑ TT Trinidad and Tobago .....
- ☑ TZ United Republic of Tanzania
- ☑ UA Ukraine .....
- ☑ UG Uganda .....
- ☑ US United States of America .....
- ☑ UZ Uzbekistan .....
- ☑ VN Viet Nam .....
- ☑ YU Yugoslavia .....
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Sheet No. ....4..

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 27 JULY 1999 (27/07/99)	[10] 1999-30517	KR		
item (2)				
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This international application contains the following **number of sheets**:

request	:	4
description (excluding sequence listing part)	:	11
claims	:	2
abstract	:	[2] 1
drawings	:	4
sequence listing part of description	:	
Total number of sheets	:	[23] 22

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Figure of the drawings which should accompany the abstract: FIG.1

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KOH, YOUNG-HOE

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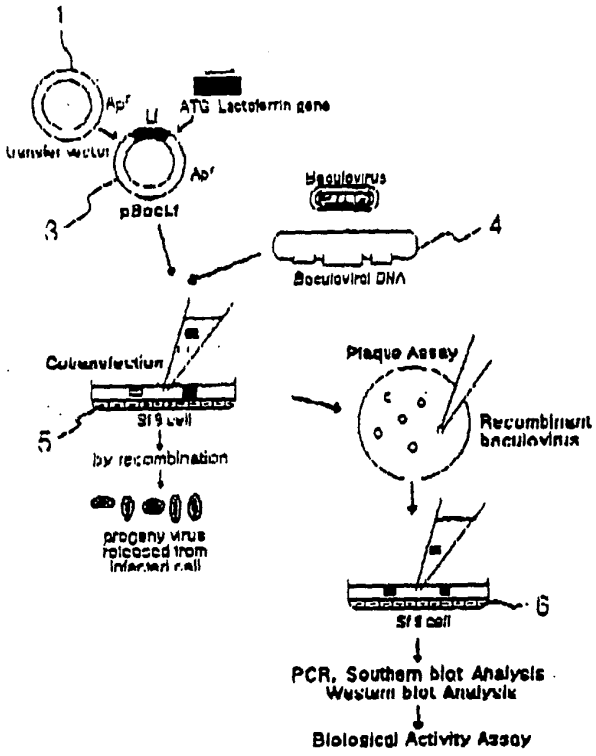
(54) Title: A HUMAN *LACTOFERRIN* PRODUCED BY USING AN INSECT CELL AND METHOD USING THE SAME



(57) Abstract: The producing process comprises Lactoferrin gene's producing recombined expression vector (pBacLf) (3) which is adjusted polyhedrin promoter in vector (pBacPAK) by combining transfer vector and remixed plasmid; producing recombined insect cell (Sf-Lf) (6) by cotransfection of the recombined revealed vector with help vector (pBacPAK) (6) at insect-cell in a culture medium; producing recombined insect virus from the recombined insect cell; and producing human Lactoferrin from the recombined insect-cell. In the invention, biological verifying method of recombined human Lactoferrin is measuring extinct rate of disease-causing germs after extracting recombined human Lactoferrin from recombined insect-cell and mixing it with disease-causing bacteria like *Pseudomonas cepacia, Pseudomonas putida, Pseudomonas fluorescence, Salmonella typhimurium, E. coli.*

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A HUMAN *LACTOFERRIN* PRODUCED BY USING AN INSECT CELL AND METHOD USING THE SAME

TECHNICAL FIELD

A present invention relates to human *lactoferrin* produced by using an insect cell and a method producing the same. More particularly, the present invention relates to human *lactoferrin* produced by using an insect cell and a method producing the same, in which the method includes the steps of transducing a human *lactoferrin* gene into an insect cell by gene recombination, cloning and expressing human *lactoferrin* in the insect cell, and producing the human *lactoferrin* by using the insect cell.

Human *lactoferrin* is a member of the transferrin family of iron-binding monomeric glycoproteins and also called "*lactotransferrin*". Such a human *lactoferrin* exists in milk of mammals including human milk, tears, saliva, mucosal secretions, and the secondary granules of polymorpho-nuclear leucocytes. It was first discovered by Peter Sorenson in 1939 and initially named "red protein" of human milk.

The *lactoferrin* (Lf) was isolated and purified from cow's milk at the first time. Since the first discovery, Lf has been isolated and purified in milk of other mammals such as human being, mouse, goat, rabbit, dog, etc. Human milk has a high content of the *lactoferrin*, for example, in the range of 6 to 8 mg/ml during the colostral phase. However, the *lactoferrin* content in human milk is decreased to about 2 mg/ml in the lactiferous phase. If the human milk is infected with bacteria during the lactiferous phase, the *lactoferrin* content in the infected milk is abruptly raised more than 30 times as high as the normal *lactoferrin* content.

Human *lactoferrin* (hLf) is a glycoprotein whose molecular weight is 78 kDa, composed of a single polypeptide chain containing 691 amino acids. The single polypeptide chain is composed of a 2-fold internal repeating unit with two folded globular

lobes. That is, human *lactoferrin* (hLf) has C and N lobes constituting C and N terminals, respectively. The two lobes have the very similar structure with a high degree of homology (more than about 40%) between the C and N terminals. With the recent advance of X-ray chrystallography, the three-dimensional structure of *lactoferrin* has been determined such that each of the C and N terminals has a site to bind one iron with high affinity and that one *lactoferrin* molecule reversibly binds two ferric ions (Fe^{+3}) (Anderson et al., 1989). Such a *lactoferrin* is present in either iron-free (i.e., apo-type) or iron-saturated state (i.e., holo-type) depending on whether it binds irons, which in turn determines the biological properties of *lactoferrin*. The apo-type *lactoferrin* is present in normal human milk. All kinds of *lactoferrin* are almost stable under the acid condition in relation to *transferrin* and releases irons at a defined pH value.

Lactoferrin, which is one of non-immunoglobulin protective proteins secreted from exocrine glands, directly or indirectly participates in the anti-bacterial mechanism and thereby affects the anti-viral action. *Lactoferrin* possesses anti-bacterial activities against various microorganisms in the state of *in vitro* and *in vivo*. Especially, *lactoferrin* in the iron-free state (apo-type) has anti-microbial activities against gram-negative bacteria such as *E. coli*, *Kleabsiela pnumoni* and *Aerobacter aerogenes*, because it chelates with Fe^{3+} ions necessary for the microorganisms and thus inhibits the growth of the microorganisms. According to an *in vitro* experiment, it has been demonstrated that *lactoferrin* had a potent anti-bacterial activity like antibiotics against 99.99% of bacterium such as *Bacillus*, *E. coli* and *Salmonella* in one hour. *Lactoferrin* also participates in mechanisms involving an iron-binding action to inhibit the growth of microorganisms and abruptly deteriorate the bacteria viability. In the mechanisms, *lactoferrin* not only damages the outer membrane of gram-negative bacteria to release a large amount of lipopolysaccaride constituting the outer membrane and thereby destroy the permeability barrier of the membrane but also increases the sensitivity of the microorganisms to

hydrophobic antibiotics such as lysozyme or rifampicin, thus reducing the resistance of the microorganisms to antibiotics. In spite of the anti-bacterial action against pathogenic organisms, it is reported that *lactoferrin* has no anti-bacterial action against bacterium beneficial to human such as *Lactobacillus* or *Bifidus*. Thus *lactoferrin* contributes to protection of newborn babies against microbial infection. Furthermore, *lactoferrin* is also found in a small amount in blood and secreted from neutrophil. Such a *lactoferrin* is a principal constituent of the secondary granules of the neutrophil and is secreted in a large amount in an inflammatory response. There are some cases that the *lactoferrin* directly has a synergy effect with lysozyme or IgA in living bodies against infected pathogenic microorganisms. As the *lactoferrin* plays an important role in the protective mechanism against infectious hosts, patients who cannot produce *lactoferrin* in the body are seriously deteriorated in resistance to various diseases with an increase in the possibility of infection with bacteria or fungi. Besides, the *lactoferrin* serves as a mediator in cell proliferation or iron transport absorption.

BACKGROUND ART

Despite that *lactoferrin* has the various functions as previously described, many studies have not been made on the human *lactoferrin* because only a small amount of *lactoferrin* is contained in blood or other bodily fluids and there is a limitation in the available quantity of colostrums sample abundant in *lactoferrin*. As human milk is regarded as a great importance, careful studies on the *lactoferrin* are being made to use a genetic engineering approach to establish the base for industrial application of microorganisms in regard to cloning and expression of human *lactoferrin* (hLf) DNA in microorganisms. However, as described above, there is still a difficulty in using *E. coli* used widely in genetic engineering as an expression strain for human *lactoferrin* (hLf) unless a special recombinant plasmid for *E. coli* is produced.

It is also of great importance to verify the biological activity of the produced recombinant *lactoferrin*, since the normal bacteria or yeasts are mostly improper as a host due to the anti-bacterial activity of *lactoferrin* itself and, if used as a host, inadequate to industrial uses in such a small quantity available. To solve this problem, Ward et al. (1992) successfully produced the recombinant *lactoferrin* using fungi such as *Aspergillus indulans* or *oryzae*. But, there was a limitation in that the *lactoferrin* was expressed in such a small amount of 5 to 25 mg/l in relation to the *lactoferrin* expressed from an insect cell. Furthermore, because the recombinant *lactoferrin* produced from the fungi was analyzed for its biological activity only in terms of affinity with Fe^{58} labeled with radioactive isotopes, there was still remained a question as to whether the recombinant *lactoferrin* actually had an anti-bacterial action on the pathogenic microorganisms.

To overcome the above problems, the present inventor has contrived a novel method for mass-production of human *lactoferrin* in a simple way and a verification method for the biological activity of the recombinant human *lactoferrin*.

It is, therefore, an object of the present invention to provide a method for producing human *lactoferrin* using an insect cell.

It is another object of the present invention to provide a recombinant insect cell for producing a human *lactoferrin* protein.

It is still another object of the present invention to provide a biological verification method for a recombinant *lactoferrin* produced from an insect cell.

DISCLOSURE OF INVENTION

The present invention relates to a method for producing human *lactoferrin* comprising the steps of transducing a human lactoferrin gene into an insect cell by gene recombination, cloning and expressing human lactoferrin in the insect cell, and using the insect cell to produce the human lactoferrin. The present invention also relates to a

verification method for the anti-bacterial activity of the recombinant human *lactoferrin*.

Reference will now be made to Figs. 1 and 2 as to a method for producing human *lactoferrin* using an insect cell according to the present invention.

The novel method for producing human *lactoferrin* by using an insect cell comprises the following steps: (a) combining a transfer vector 1 with a recombinant plasmid phLf-8 2 to prepare a recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK; (b) cotransfecting the recombinant expression vector together with a help vector pBacPAK6 4 into an insect cell Sf9 5 in a culture medium to prepare a recombinant insect cell Sf-Lf 6, and producing a recombinant insect virus from the recombinant insect cell; and (c) producing human *lactoferrin* from the recombinant insect cell Sf-Lf 6.

In the step of producing the recombinant insect cell, the culture medium in which the recombinant insect cell has been cultured is subjected by centrifugal separation to obtain a progeny virus originated from the insect cell.

To prepare the recombinant insect virus, the transfer vector 1 is first combined with the recombinant plasmid 2 to produce the recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK, and the recombinant expression vector is cotransfected with the help vector pBacPAK6 4 into the insect cell Sf9 5 in a culture medium to produce the recombinant insect cell Sf-Lf 6, from which the recombinant insect virus is produced.

The most widely used insect cell is *Spodoptera frugiperda* (Sf9) originated from army worm. The Sf9 cell line is also used as a host cell in the present invention. The Sf9 cell line, if infected with an insect virus such as *Baculovirus*, promotes synthesis of a mucous protein called polyhedrin. This means that the polyhedrin promoter for synthesizing polyhedrin in the Baculovirus is highly activated. It is stated in the related reports that the polyhedrin was produced in the insect cell in an amount of 1 to 500 mg/l

and that the concentration of the external protein expressed depended on the type of protein or gene (Kaplan et al., 1990; Davidson et al, 1990; and Kaplan et al., 1991). The insect cell was highly analogous to the higher animal cells such as mammalian cells in terms of glycoprotein, phosphorylation, fatty acid acylation, amidation, and proteolytic processing, so that most higher animal cell proteins expressed in the insect cell had biological activities. The human *lactoferrin* as used herein was also verified to have a biological activity.

The expression vector used in the present invention is an insect virus DNA and the most widely used expression vector is multiple polyhedrosis virus AcMNPV called *Autographa califonica*. The life cycle and the infection cycle of the *Baculovirus* have been reported in the related document, King L.A and R.D. Possee, The Baculovirus Expression System, A laboratory guide, HAPMAN and HALL. As stated above, the present invention uses an expression vector (pBacPAK, Clontech) originated from *Baculovirus*.

In the present invention, there are used various biochemical and molecular biological methods in order to verify the production of human *lactoferrin* from the insect cell. For example, PCR, Southern blot analysis and Western blot analysis are performed to support a fundamental study for producing a gene recombinant *lactoferrin* in the molecular level.

According to the present invention, the recombinant *lactoferrin* produced from the insect cell is biologically verified in a new way. That is, the recombinant *lactoferrin* is extracted from the insect cell and mixed with pathogenic bacteria such as *Pseudomonas cepacia, Pseudomonas putida, Pseudomonas fluorescence,* Salmonella *typhimurium* and *E. coli*, after which it is observed how much of the pathogenic bacteria have been destroyed in one hour at maximum.

On the other hand, the recombinant insect cell Sf-Lf used in the present invention is considered as an ideal system for mass production of human *lactoferrin* because it can

be cultured in a flask due to its susceptibility of suspension culture and requires neither CO_2 unlike the higher animal cells nor fetal bovine serum (FBS) in cultivation.

BRIEF DESCRIPTION OF THE DRAWINGS

Further objects and advantages of the invention can be more fully understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a flow chart showing a process of producing a recombinant human *lactoferrin* using an insect cell according to the present invention.

Fig. 2 is a flow chart showing a process of producing a recombinant expression vector pBacLf according to the present invention.

Fig. 3 is a photograph showing the electrophoresis pattern of the recombinant expression vector pBacLf after cleavage with a restriction enzyme according to the present invention.

Fig. 4 is a photograph of the agarose gel electrophoresis pattern showing that a human *lactoferrin* cDNA has been cloned from the recombinant baculovirus DNA isolated from the recombinant insect cell Sf-Lf according to the present invention.

Fig. 5 is a Southern blot photograph showing that a human *lactoferrin* DNA was cloned from the recombinant virus DNA obtained in Fig. 4.

Fig. 6 is a Western blot photograph and SDS-PAGE photograph showing that the recombinant insect cell Sf-Lf expresses and produces the human *lactoferrin* protein.

BEST MODE FOR CARRYING OUT THE INVENTION

EXAMPLE 1: Cultivation of Insect Cell and Production of Recombinant Expression Vector pBacLf

A *Spodoptera frugiperda* ovary cell Sf9 was used as an insect cell and cultured at a

low temperature of 28 °C. The insect cell Sf9 was infected with an *autographa californica* nuclear polyhedrosis virus (AcMNPV) (pBacPAK 6) and cultured in a Grace's medium containing 10% FBS, lactalbumine, hydrolysate and antimycotic antibiotics. The insect cell was commercially available from Invitrogen Inc. (PO Box 2312,9704CH Groningen, Netherlands). The virus, AcMNPV (pBacPAK6) was provided by Clontech laboratories Inc. (1020 East Meadow Circle, Palo Alto, C.A. 94303-4230, USA).

To transfer a *lactoferrin* gene into a baculovirus gene, a 2.1 kb full gene including the start codon and the signal sequence of the *lactoferrin* was produced from the existing recombinant plasmid for cloning a transfer vector (pBacPAK8, Clontech Co.) including the polyhedrin promoter site (5.5 kb), and then inserted into *E. coli* in the same direction of the polyhedrin promoter to prepare a recombinant expression vector. The transfer vector (pBacPAK8) was commercially available from Clontech laboratories Inc. (1020 East Meadow Circle, Palo Alto, C.A. 94303-4230, USA). The recombinant expression vector thus obtained was treated with a restriction enzyme to verify the *lactoferrin* gene, which was named "pBacLf". A process for producing the expression vector pBacLf is illustrated in Fig. 2.

Fig. 3 is a photograph showing the electrophoresis pattern of a selected recombinant expression vector pBacLf after cleavage with a restriction enzyme, wherein lane 1 is a size maker (λ/BstE I); lane 2 is the super coil of the recombinant expression vector pBacLf; lane 3 is the recombinant expression vector pBacLf cleaved with restriction enzymes BamH and Not I to verify the cleaved *lactoferrin* gene at 2.1 kb in terms of the full size of *lactoferrin*; lane 4 is the recombinant expression vector treated with a restriction enzyme, Eco R V to verify the *lactoferrin* gene; lane 5 is the recombinant expression vector treated with a restriction enzyme, Sma I to verify the *lactoferrin* gene; lane 6 is the recombinant expression vector treated with a restriction enzyme, Bgl II to verify the *lactoferrin* gene; lane 7 is the recombinant expression vector

treated with a restriction enzyme, Pst I to verify the *lactoferrin* gene; and lane 8 is a size marker. As shown in Fig. 3, a human *interferrin* gene shows at 2.1 kb when the recombinant expression vector was cleaved with restriction enzymes BamH I and Not I into a fragment

EXAMPLE 2: Selection of a Recombinant Insect Cell Sf-Lf and Identification of a Recombinant Virus

Sf9 cells were inoculated in an amount of about 1.0×10^6 cells in a Grace's basic medium containing 10% FBS and were cultured for 4 hours. The insect cell was washed with the Grace's basic medium twice and remained at the ambient temperature for 30minutes. A mixture of a virus DNA (BacPAK 6, Clontech Co.) and a recombinant transfer vector prepared for liposome-mediated transfection, together with lipofectin was dropped on a cell monolayer. The mixture was added to the Grace's medium containing serum and antibiotics and was cultured at 28 °C for 5 days. The supertant was diluted with the culture solution in ten stages, 3 to 5 times and was inoculated into the insect cell Sf9 cultured in a monolayer on a 60mm-diameter plate. When the virus was adsorbed, the dissolved agarose-containing medium was hardened on the insect cell. After 6 to 7 days, the insect cell with the agarose-containing medium was dyed with neutral red, which dyes dead cells distinguished and forms a plaque. A microscope was used to select a plaque in which the polyhedrin by the infection with the recombinant virus was not formed. The plaque with the agarose was sucked up with a Pasteur pipette and was suspended in a 1ml medium. In order to verify whether the recombinant virus contains the *lactoferrin* gene, another insect cell Sf9 cultured in a new Grace's medium was infected with the recombinant virus and the recombinant virus DNA was isolated, after which the electrophoresis patterns of the isolated DNA were compared by agarose gel electrophoresis. By polymerase chain reaction (PCR) using a primer capable of amplifying *lactoferrin*, it was identified and after treatment with a restriction enzyme, the *lactoferrin*

gene (2.1 kb) was identified by Southern blot analysis.

Fig. 4 shows that the *lactoferrin* cDNA (2.1 kb) was cloned from the recombinant virus DNA isolated from the recombinant insect cell Sf-Lf, in which lane 1 shows a size marker (λ/BstE II); lane 2 is a negative control (pBacPAK8); and each of lane 3 and 4 is a recombinant virus DNA. It is seen from lane 3 that there has been amplified the human *lactoferrin* cDNA (2.1 kb) using a primer exclusively amplifying the human *lactoferrin* from the recombinant virus DNA.

Fig. 5 verifies the *lactoferrin* DNA (2.1 kb) by Southern blot analysis for the recombinant virus DNA treated with restriction enzymes such as BamH I, Not I and Acc I, based on the fact verified in Fig. 4, in which lane 1 shows a recombinant virus intact DNA; lane 2 is a recombinant virus DNA (treated with BamH I/Not I); lane 3 is a recombinant virus DNA (treated with Acc I); lane 4 is a recombinant virus DNA (treated with BamH I/Not I/Bgl II); lane 5 is an amplified PCR *lactoferrin* gene produced from the virus DNA; lane 6 is a DIG-labeled size marker; lane 7 is a super coil DNA of the recombinant plasmid (pBacPAK8); and lane 8 is a negative control (pGEMLf) (treated with BamH I/Not I).

The negative control was pBacPAK8 and the positive control was lactoferrin-gene-containing pGEMLf cleaved with a restriction enzyme BamH I/Not I. The probe as used herein was a part of the N-lobe of DIG-labeled *lactoferrin* cDNA. As shown in Fig. 5, the band was observed at the same location (2.1 kb) of the positive control in the recombinant baculovirus DNA. It demonstrates that the selected recombinant virus of the present invention contained human *lactoferrin* DNA.

EXAMPLE 3: Expression of Human *Lactoferrin* from Recombinant Insect Cell (Sf-Lf) and its Verification

To verify expression of protein, the recombinant cell was pulverized with a cell

lysis buffer (50 mM Tris-HCl, pH 8.0, 5% 2-mercaptoethanol, 0.4% w/v SDS, 10 mM EDTA) and applied to coomassie-blue polyacrylamide gel running, after which a Western blot analysis was performed using anti-*lactoferrin* (anti-Lf).

As shown in Fig. 6, the recombinant viral stock was inoculated into the insect cell Sf9 and the cells were pulverized with the insect host cell Sf9 at the fourth day. The supernatant was collected for a Western blot analysis with anti-*lactoferrin* (anti-Lf) Ab and SDS-PAGE and, as a result of which the band was color-developed at the same location (80 kDa) of colostrums used as a positive control and purified *lactoferrin* protein. This demonstrates that the human *lactoferrin* protein was produced in the recombinant insect cell (Sf-Lf). Lane 1 shows a protein size marker, lane 2 is a colostral soup, lane 3 is the insect cell Sf9, lanes 4 and 5 are the recombinant insect cells. A densitometry revealed that the expressed *lactoferrin* was in an amount of more than 800 mg/l. The amount expressed is much higher than the amount of *lactoferrin* expressed from *Aspergillus Nidulans* or *Aspergillus Oryzae* and shows economically high productivity.

EXAMPLE 4: Verification of Anti-bacterial Activity of Recombinant *Lactoferrin*

To measure the anti-bacterial activity of the recombinant insect cell (Sf-Lf) against pathogenic microorganisms, the insect cell (Sf-Lf) was pulverized by a freeze and thaw method and the supernatant was mixed with the pathogenic microorganisms in the *lactoferrin* concentration of about 250 μg/ml. The mixture was then smeared to a plate count agar plate at intervals of 0, 15, 30, 45 and 60 minutes.

It can be seen from the cell count of Table 1 that the *lactoferrin*-containing the supertant alone destroys the pathogenic microorganisms within one hour. For the negative control, insect cell (Sf) was pulverized and subjected to microassay on pathogenic microorganisms in the same way as for the recombinant insect cell (Sf-Lf), in which case there was no decrease in the number of cells.

The above results revealed that the *lactoferrin* protein produced from the

recombinant insect cell (Sf-Lf) possessed an anti-bacterial activity.

INDUSTRIAL APPLICABILTY

The recombinant insect cell (Sf-Lf) produced by the method for producing human *lactoferrin* using an insect cell according to the present invention is advantageous in that it can be cultured in a flask due to its susceptibility of suspension culture, requiring neither carbon dioxide (CO_2) unlike the higher animal cells, nor fetal bovine serum (FBS) in the culture, as a result of which using the recombinant insect cell allows mass production of human *lactoferrin* at a low cost in a simple way.

Table 1

Strain	Cell Count (cfu/ml)									
	*E. coli 300		*S. typhimurium 114		@P. putida		@P. fluorescence		@P. cepasia 9613	
	-	+	-	+	-	+	-	+	-	+
0 min	$>10^7$	$>10^7$	$>10^7$	$>10^7$	$>10^7$	6.7×10^6	$>10^7$	1.8×10^6	5×10^3	1.4×10^3
15 min	$>10^7$	$>10^5$	$>10^7$	5.0×10^4	$>10^7$	3.0×10^4	$>10^7$	1.4×10^4	5×10^3	9.8×10^2
30 min	$>10^7$	1.3×10^4	$>10^7$	1.2×10^4	$>10^7$	6.9×10^3	$>10^7$	4.0×10^3	5×10^3	2.1×10^2
45 min	$>10^7$	6.2×10^3	$>10^7$	2.0×10^3	$>10^7$	3.0×10^3	$>10^7$	2.9×10^3	5×10^3	10
60 min	$>10^7$	10^2	$>10^7$	1.2×10^2	$>10^7$	10^2	$>10^7$	5×10^2	5×10^3	0

Note: * indicates an animal pathogenic bacterium; @ indicates a food contaminant bacterium; + indicates a culture with *lactoferrin*; and - indicates a culture without *lactoferrin*

CLAIMS

1. A method for producing human *lactoferrin* by using an insect cell comprising the steps of:

(a) combining a transfer vector 1 with a recombinant plasmid phLf-8 2 to produce a recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK;

(b) cotransfecting said recombinant expression vector together with a help vector pBacPAK6 4 into an insect cell Sf9 5 in a culture medium to produce a recombinant insect cell Sf-Lf 6, and producing a recombinant insect virus from said recombinant insect cell; and

(c) producing human *lactoferrin* from said recombinant insect cell Sf-Lf 6.

2. The method of claim 1, wherein said producing a recombinant insect virus step further comprises the step of performing a centrifugal separation of the culture medium containing the recombinant insect cell cultured in the producing step (b) to obtain a progeny virus from the insect cell contained in the upper layer.

3. A human *lactoferrin* produced by a method comprising the steps of:

(a) combining a transfer vector 1 with a recombinant plasmid phLf-8 2 to produce a recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK;

(b) cotransfecting said recombinant expression vector together with a help vector pBacPAK6 4 into an insect cell Sf9 5 in a culture medium to produce a recombinant insect cell Sf-Lf 6, and producing a recombinant insect virus from said recombinant insect cell; and

(c) producing human *lactoferrin* from said recombinant insect cell Sf-Lf.

4. A recombinant insect virus produced by a method comprising the steps of:

(a) combining a transfer vector 1 with a recombinant plasmid phLf-8 2 to produce a recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK;

(b) cotransfecting said recombinant expression vector together with a help vector pBacPAK6 4 into an insect cell Sf9 5 in a culture medium to produce and culture a recombinant insect cell Sf-Lf 6; and

(c) producing a recombinant insect virus from said recombinant insect cell Sf-Lf.

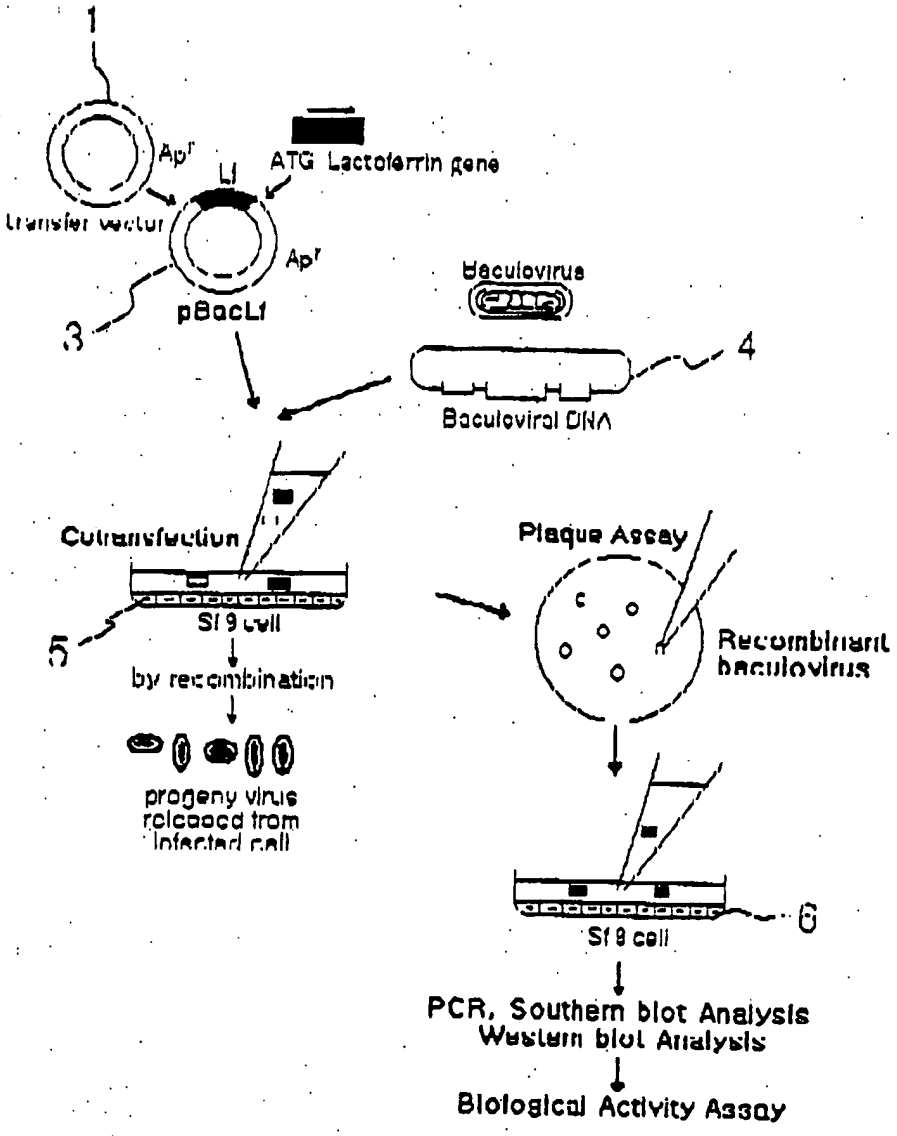
5. A biological verification method for a recombinant human *lactoferrin*, comprising the steps of:

mixing human *lactoferrin* produced by the method of claim 1 with a pathogenic microorganism; and,

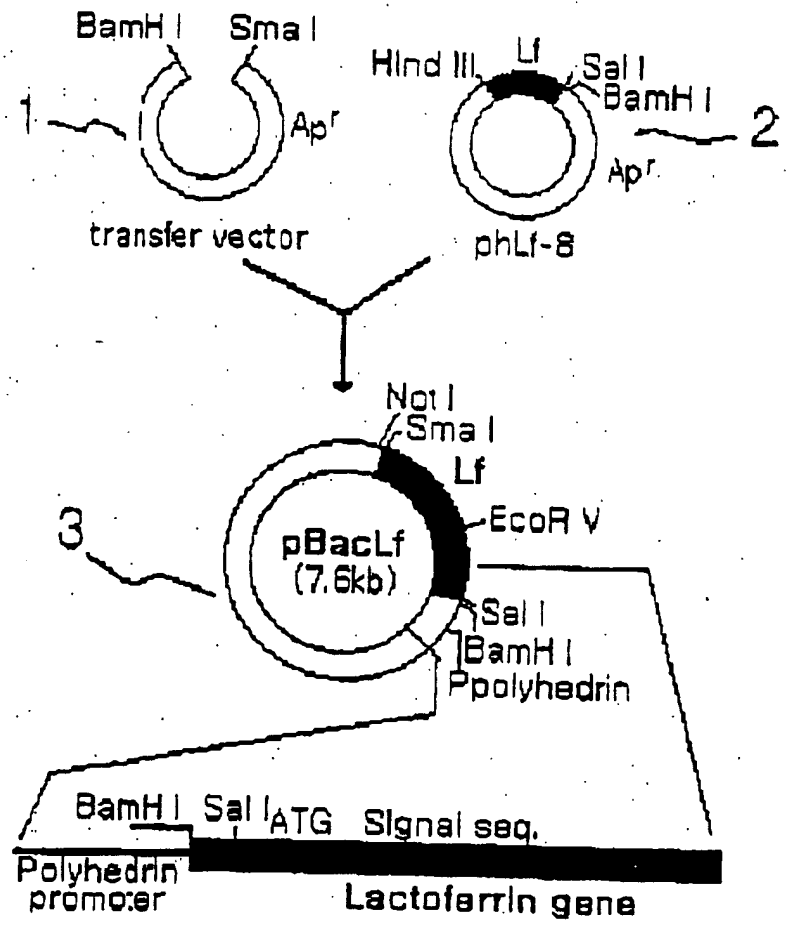
measuring anti-bacterial activity of said mixture against the pathogenic microorganism.

6. The method of claim 5, wherein said pathogenic microorganism is selected from the group consisting of *Pseudomonas cepacia, Pseudomonas putida, Salmonella typhimurium*, *Pseudomonas fluorescence* and *E. coli*.

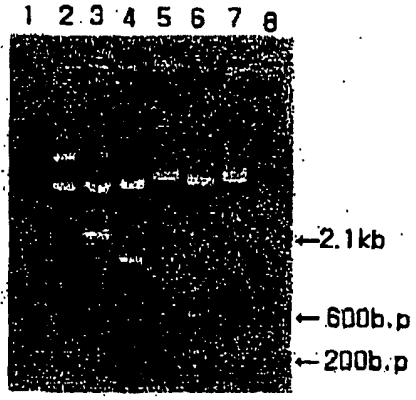
【Fig. 1】


1
Ap'
LI
ATG Lactoferrin gene
Transfer vector
Ap'
pBacLf
3
Baculovirus
4
Baculoviral DNA
Cotransfection
Sf 9 cell
5
by recombination
progeny virus
released from
infected cell
Plaque Assay
Recombinant
baculovirus
Sf 9 cell
6
PCR, Southern blot Analysis
Western blot Analysis
Biological Activity Assay

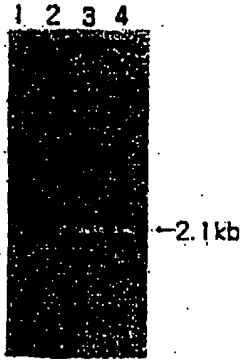
[Fig. 2]


BamH I
Sma I
Apr
1
transfer vector
Hind III
Lf
Sal I
BamH I
Apr
2
phLf-8
Not I
Sma I
Lf
EcoR V
3
pBacLf
(7.6kb)
Sal I
BamH I
Ppolyhedrin
BamH I
Sal I
ATG
Signal seq.
Polyhedrin
promoter
Lactoferrin gene

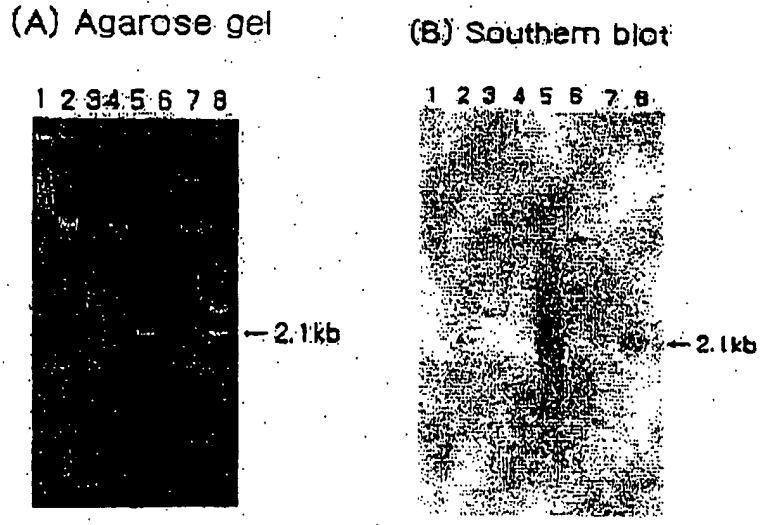
【Fig. 3】



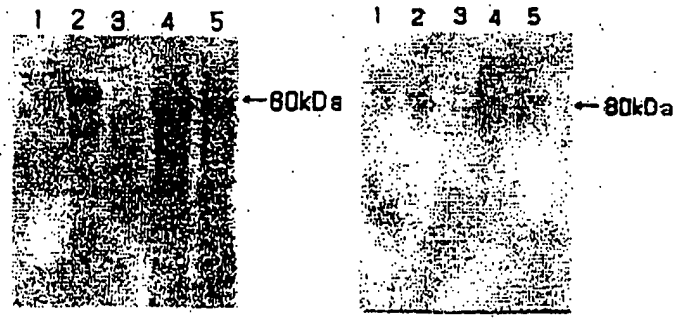
【Fig. 4】



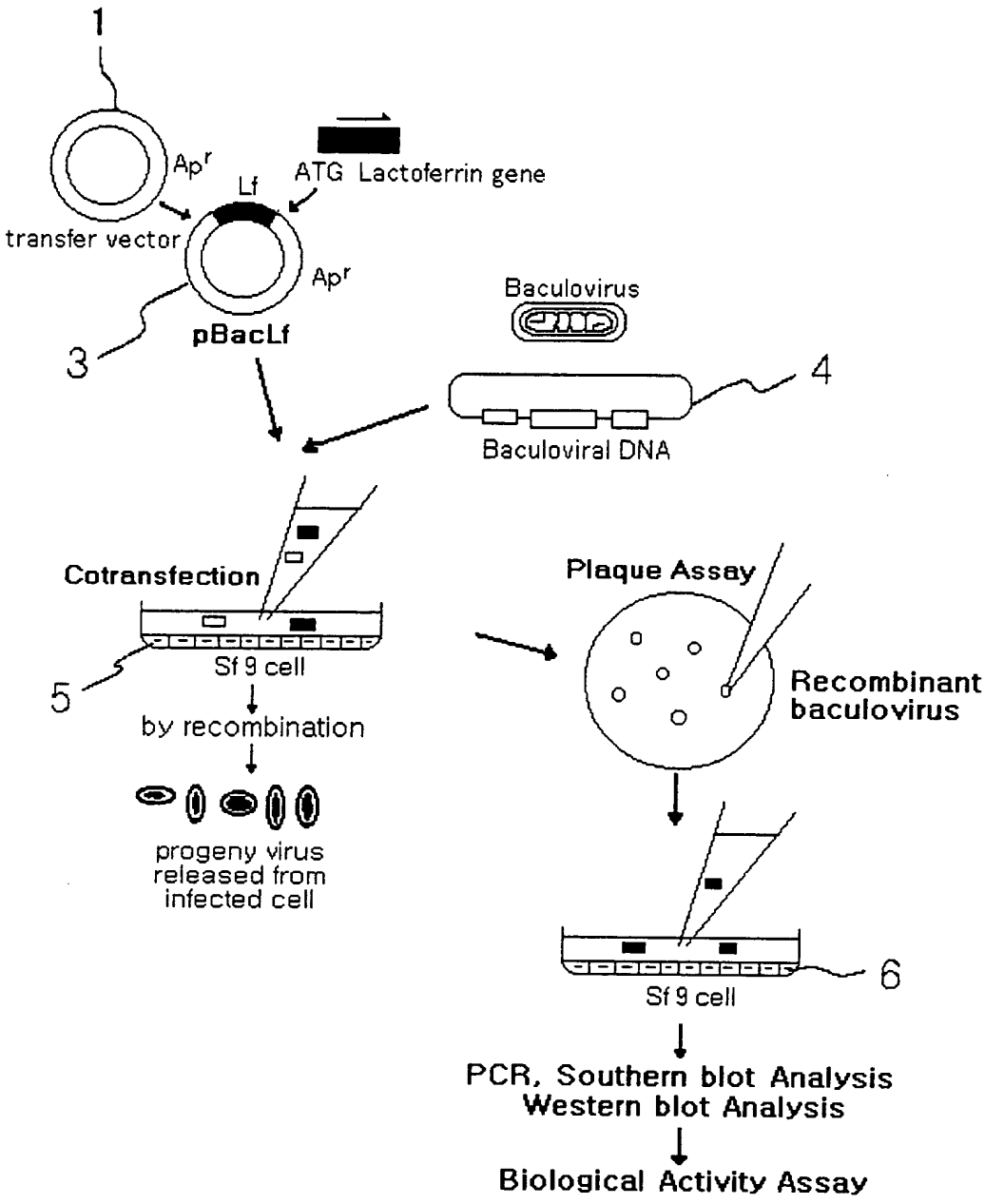
[Fig. 5]


(A) Agarose gel
(B) Southern blot
1 2 3 4 5 6 7 8
2.1kb
1 2 3 4 5 6 7 8
2.1kb

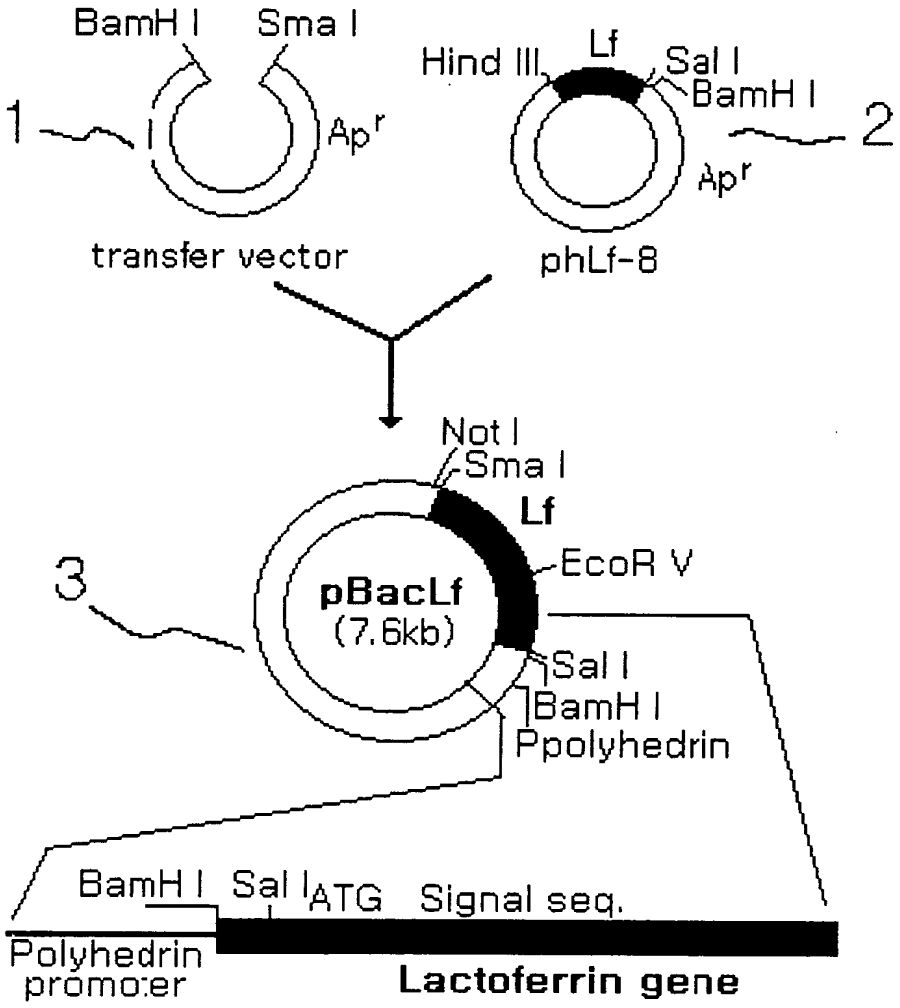
[Fig. 6]


1 2 3 4 5
80kDa
1 2 3 4 5
80kDa

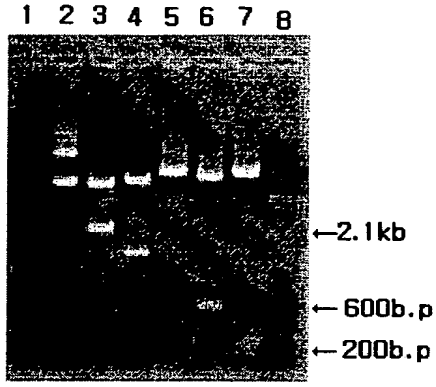
【도 1】


1
Ap^r
Lf
ATG Lactoferrin gene
transfer vector
Ap^r
pBacLf
3
Baculovirus
4
Baculoviral DNA
Cotransfection
Sf 9 cell
5
by recombination
progeny virus
released from
infected cell
Plaque Assay
Recombinant
baculovirus
Sf 9 cell
6
PCR, Southern blot Analysis
Western blot Analysis
Biological Activity Assay

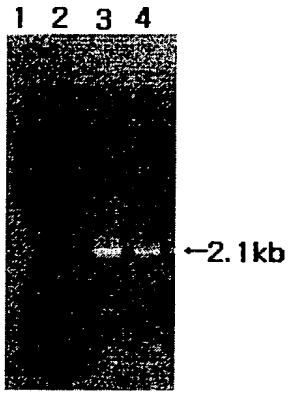
【도 2】


BamH I
Sma I
1
Ap^r
transfer vector
Hind III
Lf
Sal I
BamH I
2
Ap^r
phLf-8
Not I
Sma I
Lf
EcoR V
3
pBacLf
(7.6kb)
Sal I
BamH I
Ppolyhedrin
BamH I
Sal I
ATG
Signal seq.
Polyhedrin
promoter
Lactoferrin gene

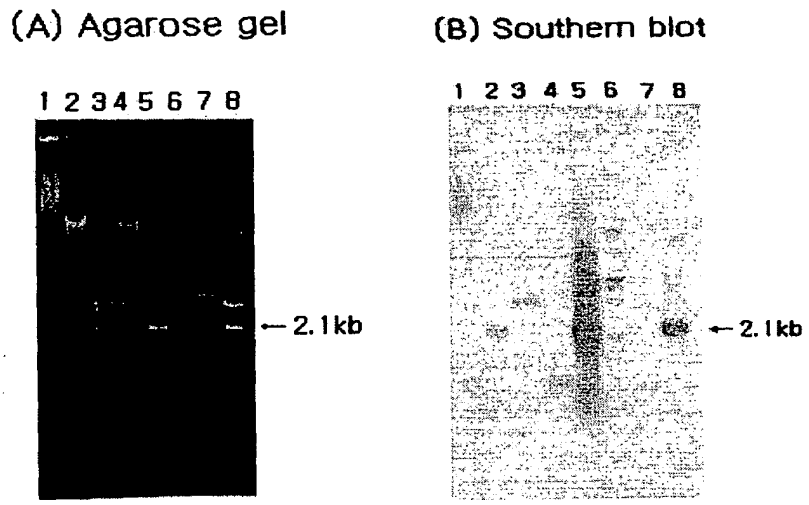
【도 3】



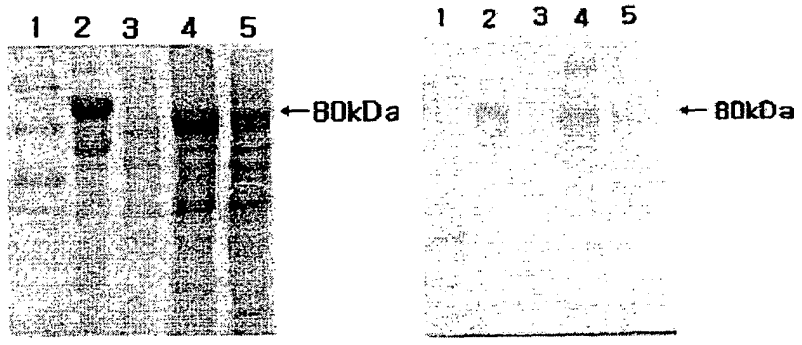
【도 4】



【도 5】


(A) Agarose gel
(B) Southern blot
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
← 2.1kb
← 2.1kb

【도 6】


1 2 3 4 5
1 2 3 4 5
←80kDa
← 80kDa

【명세서】

【발명의 명칭】

곤충세포를 이용하여 제조된 사람 락토페린 및 그 제조방법

【기술 분야】

본 발명은 곤충세포를 이용하여 제조된 사람 락토페린(Human lactoferrin) 및 그 제조방법에 관한 것이다. 더욱 구체적으로 본 발명은 유전자 재조합에 의해 사람 락토페린 유전자를 곤충세포에 형질도입하고, 이를 배양하여 발현시킨 후 이로부터 사람 락토페린을 생성하는 곤충세포를 이용하여 제조된 사람 락토페린 및 그 제조방법에 관한 것이다.

사람 락토페린은 철(iron)과 결합하는 당 단백질인 트랜스페린(transferrin)의 일종으로서 락토트랜스페린(lactotransferrin) 이라고도 한다. 주로 모유, 우유, 침, 눈물, 점액분비물과 다형핵 류코사이트(polymorpho-nuclear leucocytes)의 제2 과립세포(secondary granules) 등에 존재하는데, 1939년 피터 소렌슨(Peter Sorenson)에 의해 처음 발견되었으며, 초기에는 인유(人乳)의 '레드 프로테인(red protein)'으로 명명되었었다.

최초의 락토페린(Lf)은 소의 유즙으로부터 정제 동정되었고, 이후에는 쥐, 염소, 토끼, 개, 사람 등 여러 포유동물의 유즙에서도 분리 정제되었다. 락토페린의 함량은 유즙에서 상대적으로 높은데, 특히 초유기간 동안에는 보통 6-8 ㎎/㎖의 함량을 유지한다. 그러나 초유를 거친 모유단계에 이르러서는 2 ㎎/㎖정도로 감소된다. 만일 이 기간동안에 세균에 감염되면 락토페린은 정상적인 농도의 30배 이상으로 급증한다.

사람 락토페린(hLf)은 분자량이 78kDa으로 691개의 아미노산(amino acid)으로 된 단일 폴리펩타이드 체인(single polypeptide chain)으로 구성되어 있다. 상기 단일 폴리펩타이드 체인은 2개의 글로뷸라 로브(globular lobes)로 폴드(fold)되어 있는 2-fold internal repeat로 구성되어 있다. 즉, 사람 락토페린(hLf)은 N-말단 부분을 구성하고 있는 N-로브와 C-말단 부분을 구성하고 있는 C-로브로 되어있다. 이 두 로브는 매우 유사한 구조를 가지고 있어서, N-말단과 C-말단 사이에는 약40% 이상의 아미노산 시퀀스 호몰로지(homology)를 갖는다. 최근 들어 엑스-레이 크리스탈로그라피(x-ray chrystallography)에 의해 락토페린의 3차 구조가 밝혀짐에 따라 락토페린은

N-말단과 C-말단에 각각 하나의 철과 결합할 수 있는 부위가 있어서 한 분자당 두개의 Fe^{3+}이온이 가역적으로 결합할 수 있음을 알게되었다(Anderson 등, 1989). 또한, 이러한 락토페린은 철(iron)의 결합유무에 따라 iron-free(apo type) 상태와 iron-saturated(holo type) 상태로 존재하게 되는데, 락토페린의 생물학적 특성은 철의 결합에 의해 영향을 받게된다. 보통 모유에는 apo-type의 락토페린이 존재하며, 모든 락토페린은 트랜스페린(transferrin)에 비해 산성(acidic) 상태에서 안정성을 갖고 pH의 영향을 받아 철을 방출시킨다.

락토페린은 외분비선(exocrine glands)에서 분비되는 비-면역글로블린 보호 단백질(non-immunoglobulin protective protein) 중의 하나로서 직접 또는 간접적으로 항균작용(anti-bacterial action)을 일으키며, 항 바이러스에 대해서도 영향을 미친다. 락토페린은 *in vitro*와 *in vivo* 중 어느 상태에서도 여러 종류의 미생물에게 광범위한 항균작용을 일으킨다. 이러한 락토페린은 iron-free state(apo-type)에서 이 콜라이(E. coli)나 클리브시엘라 뉴모니아(*Kleabsiella pneumonia*), 에어로벡터 에어로진(*Aerobacter aerogenes*) 등의 그램-음성 박테리아(Gram-negative bacteria)에 대해 높은 항미생물활성(anti-microbial activities)을 갖는데, 이는 락토페린이 미생물이 필요로 하는 Fe^{3+}이온을 빼앗아 킬레이팅(chelating) 시킴으로서 미생물의 생장을 억제하기 때문이다. 실제로 *in vitro* 실험결과, 락토페린은 바실루스(*Bacillus*), 이 콜라이(*E. coli*), 살모넬라(*Salmonella*) 등의 균을 1시간 이내에 99.99% 사멸시켜 항생물질에 뒤지지 않는 살균작용을 보였다. 또한, 락토페린은 철을 빼앗아 미생물의 성장을 억제시키는 작용 기작과 박테리아 생육성(bacteria viability)을 급속하게 감소시키는 작용 기작을 갖고있다. 상기 기작은 락토페린이 그램 음성균(Gram-negative bacteria)의 외막(outer membrane)에 손상을 주어 외막의 구성성분인 당지질(lipopolysaccharide)을 다량 방출(release)시킴으로써 투과장벽(permeability barrier)을 파괴하고, 리소자임(lysozyme)이나 리팜피신(rifampicin)같은 소수성 항생제(hydrophobic antibiotics)에 대한 민감성을 증대시켜 저항성을 잃게 하여 사멸되게 한다. 그러나 이렇게 병원체에 대한 살균작용을 갖는 락토페린이 락토바실러스(Lactobacillus)나 비피더스(Bifidus)와 같은 인체에 유용한 세균에 대해서는 항균작용을 일으키지 않는다는 보고가 있다. 따라서, 이렇게 독특한 물질인 락토페린은 세균이나 바이러스 감염으로부터 면역에 미숙한 신생아를 지켜주는 역할을 한다. 락토페린은 혈액 내에도

소량이 포함되어 있는데, 대부분 호중구(neutrophil)에서 분비(secretion)된다. 락토페린은 호중구의 제2 과립세포의 주된 구성성분으로서 염증반응(inflammatory response) 시에 다량 분비된다. 락토페린은 종종 체내에서 리소자임(lysozyme)이나 IgA와 함께 감염된 병원성 미생물에게 직접 작용하여 파괴시킴으로써 미생물 사멸에 상승효과를 주기도 한다. 이와 같이 락토페린은 숙주에 대한 방어 기작에서 매우 중요한 역할을 담당하고 있으므로, 락토페린을 생성하지 못하는 환자는 각종 질병에 대해 저항성이 훨씬 떨어지고 세균과 곰팡이에 대하여 감염이 증가한다. 그 외에 락토페린은 세포 증식(cell proliferation)이나 철의 전달흡수(iron transport absorption) 등에서도 매개체로서의 역할을 담당한다.

【배경 기술】

이러한 락토페린의 많은 작용에도 불구하고 사람 락토페린에 대한 연구는 아직 미미한 편이다. 이것은 락토페린이 혈액이나 그 밖의 다른 체액 내에 존재하지만 그 양이 매우 미미하고, 초유(colostral whey)에 상당량이 존재하더라도 초유를 얻을 수 있는 시료에 한계가 있기 때문이다. 최근에는 모유의 중요성이 강조되어 락토페린에 대한 연구가 활성화되고 미생물을 이용한 산업적 응용의 기본토대가 이루어지도록 유전 공학적 방법을 적용하여 사람 락토페린(hLf) DNA를 미생물에 클로닝하고 발현(expression) 시키고자 한다. 그러나 앞에서 언급한 바와 같이 사람 락토페린은 유전 공학에 많이 사용하고 있는 이 콜라이(E. coli) 등을 위한 특별한 재조합 플라스미드가 제작되어지지 않는 한, 이 콜라이를 발현균주로 사용하는 것은 대단히 어려운 일이다.

또한, 생성된 재조합 락토페린의 생물학적 활성의 검증을 하는 것은 매우 중요하다. 왜냐하면, 락토페린 자체가 가지는 항균성으로 인해 일반적으로 사용되는 박테리아나, 효모 등이 대부분 숙주로서 부적합하거나, 혹은 사용된다 하더라도 생성 양이 극히 적어 산업적으로 사용하기가 부적절하기 때문이다. 이를 타개하기 위하여 와드(Ward) 등(1992)은 곰팡이인 *Aspergillus nidulans*나 *oryzae*를 사용하여 재조합 락토페린을 생성하는데 성공하였으나, 그 발현 양이 5-25mg/ℓ로 곤충세포(insect cell)에 비하여 아주 낮은 편이었다. 또한, 이들은 곰팡이들로부터 생성된 재조합 락토페린의 생물학적 활성을 방사선 동위원소가 표지된 Fe^{56}의 결합력으로만 검증하였기 때문에 이들 재조

합 락토페린이 실제로 항균 작용을 하는가에 대해서는 의문점이 있다.

따라서, 본 발명자는 종래 기술의 단점을 개선하여 보다 용이하게 사람 락토페린을 대량생산할 수 있는 방법 및 재조합 사람 락토페린의 생물학적 활성을 검증하는 새로운 방법을 개발하기에 이르렀다.

따라서, 본 발명의 목적은 곤충세포를 이용하여 사람 락토페린을 생산할 수 있는 방법을 제공하기 위한 것이다. 또한, 본 발명의 다른 목적은 사람 락토페린 단백질을 생산하는 재조합 곤충세포를 제공하기 위한 것이다. 뿐만 아니라, 본 발명의 또 다른 목적은 곤충세포로부터 생성된 재조합 락토페린을 생물학적으로 검증하기 위한 방법을 제공하기 위한 것이다.

【발명의 상세한 설명】

본 발명은 유전자 재조합에 의해 사람 락토페린 유전자를 곤충세포에 형질도입하고, 이를 배양하여 발현시킨 후 이로부터 사람 락토페린을 생산하는 곤충세포를 이용한 사람 락토페린 생산방법에 관한 것이다. 또한, 생산된 재조합 사람 락토페린의 항균성 검증방법에 관한 것이다.

도1 및 도2를 참조하여 본 발명의 곤충세포를 이용한 사람 락토페린 제조방법을 설명한다.

본 발명의 곤충세포를 이용한 사람 락토페린의 제조방법은 전달 벡터(tansfer vecter)(1) 및 재조합 플라스미드(phLf-8)(2)를 조합하여 락토페린 유전자가 벡터(pBacPAK) 내의 폴리헤드린(polyhedrin) 프로모터(promotor)의 조절을 받도록 변형한 재조합 발현 벡터(pBacLf)(3)를 제조하고; 상기 재조합 발현 벡터를 헬프 벡터(pBacPAK6)(4)와 함께 배양기내의 곤충세포(Sf9)(5)에 코트랜스펙션(cotransfection)하여 재조합 곤충세포(Sf-Lf)(6)를 제조하고 상기 재조합 곤충세포로부터 재조합 곤충 바이러스를 제조하고; 그리고 상기 재조합 곤충세포(Sf-Lf)(6)로부터 사람 락토페린을 생산하는 단계로 이루어진다.

상기 재조합 곤충세포 제조단계에서 재조합 곤충세포가 배양된 배양액을 원심분리하여 상층액에 존재하는 상기 곤충세포로부터 나온 자손 바이러스(progeny virus)를 얻는다.

상기 재조합 곤충 바이러스는 전달 벡터(tansfer vecter)(1)와 재조합 플라스미드(2)를 조합하여 락토페린 유전자가 벡터(pBacPAK) 내의 폴리헤드린 프로모터의 조절을 받도록 변형한 재조합 발현 벡터(pBacLf)(3)를 제조하고;

상기 재조합 발현 벡터를 헬프 벡터(pBacPAK6)(4)와 함께 배양기내의 곤충세포(Sf9)(5)에 코트랜스펙션(cotransfection)한 다음 재조합 곤충세포(Sf-Lf)(6)를 제조하여 배양하고; 그리고 상기 재조합 곤충세포(Sf-Lf)로부터 재조합 곤충 바이러스를 생산하는 단계로 이루지는 방법으로 제조된다.

일반적으로 많이 사용되는 곤충세포는 거염벌레(armyworm)로부터 유래된 Sf9 cell(*Spodoptera frugiperda*)이다. 본 발명에서도 상기 Sf9 세포주(cell line)를 숙주로 사용한다. 상기 Sf9 세포주는 세포 내에 곤충 바이러스인 바큐로바이러스(Baculovirus)에 의해 감염되면 폴리헤드린(polyhedrin)이라는 점액성 단백질을 많이 합성하며, 이는 바큐로바이러스에 있는 폴리헤드린을 합성하는 폴리헤드린 프로모터가 매우 활성화되어 작용된다는 사실을 말해준다. 지금까지 보고된 바(Kaplan 등, 1990: Davidson 등, 1990, Kaplan 등, 1991)에 의하면 곤충세포 내에서 생성된 폴리헤드린의 양은 1mg/ℓ-500mg/ℓ로 다양하며, 따라서 외부 단백질이 발현되는 농도도 단백질이나 유전자에 따라 다양하다. 상기 곤충세포는 고등 동물세포인 포유류 세포(mammalian cell)와 당단백질(glycoprotein), 인산화 반응(phosphorylation), 지방산 아실레이션(fatty acid acylation), 아미드화 반응(amidation), 단백질분해 공정(proteolytic processing) 등이 매우 유사하며, 따라서 곤충 세포에서 발현되는 대부분의 고등생물 단백질은 생물학적으로 활성을 지니고 있게 된다. 본 발명에서 사용한 사람 락토페린도 생물학적 활성을 지닌 것으로 나타난다.

본 발명에서 사용된 발현 벡터는 곤충 바이러스 DNA이며, 가장 많이 사용되는 것은 오토그라파 칼리포니카(*Autographa califonica*)로 알려진 복합 폴리헤드로시스 바이러스(multiple polyhedrosis virus)(AcMNPV)이다. 바큐로바이러스의 라이프 사이클(life cycle)이나 감염주기는 킹(King) 등의 저서(King, L.A and R.D. Possee, The Baculovirus Expression System. A laboratory guide, HAPMAN and HALL)에 자세히 기재되어 있다. 본 발명에서는 전술한 바와 같이 바큐로바이러스로부터 유래된 발현 벡터(pBacPAK, Clontech)를 사용한다.

본 발명에서는 재조합 곤충세포가 사람 락토페린을 생성하는 것을 뒷받침 하기 위하여 여러 가지 생화학적, 분자생물학적 방법을 사용한다. 예를 들면, PCR, 써던 블랏(Southern blot), 웨스턴 블랏(western blot) 등을 사용하여 분자 수준에서 유전자 재조합 락토페린을 생성하기 위한 기초적인 연구를

수행한다.

본 발명에서는 곤충세포로부터 생성된 재조합 락토페린의 생물학적 검증을 새로운 방법으로 실시한다. 즉, 재조합 락토페린을 곤충세포로부터 추출하여 슈도모나스 세파시아(*Pseudomonas cepacia*), 슈도모나스 푸티다(*Pseudomonas putida*), 슈도모나스 플루오레슨스(*Pseudomonas fluorescence*), 살모넬라 티피뮤리움(*Salmonella typhimurium*), 또는 이 콜라이(*E. coli*)와 같은 병원성 박테리아와 혼합하여 병원성균의 최대 1시간내에 사멸 정도를 관찰한다.

한편, 본 발명에서 사용된 재조합 곤충세포(Sf-Lf)는 현탁배양(suspension culture)이 가능하여 플라스크(flask) 배양이 가능하고, 일반적인 고등 생물 세포와는 달리 CO_2가 필요 없고, 배양시 FBS(Fetal Bovine Serum)를 사용하지 않아도 된다는 점에서 향 후 대량 생산을 위한 이상적인 시스템이 될 수 있다.

【도면의 간단한 설명】

도1은 본 발명의 곤충세포를 이용한 재조합 사람 락토페린의 제조공정을 나타낸 공정도이다.

도2는 본 발명의 재조합 발현 벡터(pBacLf)의 제조과정을 나타낸 공정도이다.

도3은 본 발명의 재조합 발현 벡터(pBacLf)의 제한 효소를 절단한 후의 전기영동 패턴 사진이다.

도4는 본 발명의 재조합 곤충세포(Sf-Lf)로부터 분리된 재조합 바큐로바이러스(Baculovirus) DNA로부터 사람 락토페린 cDNA가 클로닝(cloning) 됨을 나타내는 아가로스 겔 전기영동 사진이다.

도5는 도4으로부터 얻은 재조합 바이러스 DNA로부터 사람 락토페린 DNA가 클로닝되었음을 보여주는 써던 블랏(southern blot) 시험결과를 나타낸 사진이다.

도6는 재조합 곤충세포 Sf-Lf가 사람 락토페린 단백질을 발현하고 생산하는 SDS-PAGE 및 이를 확인하는 웨스턴 블랏(western blot) 시험결과를 나타낸 사진이다.

<도면의 부호에 대한 간단한 설명>

1: 전달 벡터
2: 재조합 플라스미드
3: 발현 벡터
4: 헬프 벡터
5: 곤충세포(Sf9)
6: 재조합 곤충세포(Sf-Lf)

【발명의 실시를 위한 형태】

실시예 1: 곤충세포의 배양 및 재조합 발현 벡터(pBacLf)의 제조

곤충세포로는 스포돕테라 프루기페르다 난소 세포(Spodoptera frugiperda ovary cell)(Sf9)를 사용하였으며, 28℃에서 저온 배양하였다. 상기 곤충세포(Sf9)에 감염(infection)시키기 위한 바이러스(virus)는 AcMNPV, Autographa californica nuclear polyhedrosis virus(pBacPAK 6, Clontech co.)를 사용하였으며, 10% FBS, 락트알부민(lactalbumine) 하이드롤리세이트(hydrolysate)와 항진균 항생제(antimycotic antibiotics)가 포함된 그레이스 배지(Grace's medium)에서 배양하였다. 상기 곤충세포 Sf9 cell은 네델란드 인비트로젠회사에서 구입하여 사용하였다. (Invitrogen, PO Box 2312,9704CH Groningen, Netherlands). 상기 바이러스 AcMNPV(pBacPAK6)는 미국 클론텍 회사(CLONTECH Laboratories, Inc. 1020 East Meadow Circle, Palo Alto, CA 94303-4230, USA)에서 구입하여 사용하였다.

락토페린 유전자를 바큐로바이러스 유전자 안으로 운반해주기 위해 폴리헤드린 프로모터 부분을 포함하는 5.5kb크기의 전달 벡터(pBacPAK8, Clontech Co.)에 클로닝하기 위해 기존의 재조합 플라스미드로부터 락토페린의 개시암호(start codon)와 신호서열(signal sequence)을 포함하는 2.1kb 완전 유전자(full gene)를 준비하여 폴리헤드린 프로모터(polyhedrin promoter)와 같은 방향으로 이 콜라이(E. coli)에 삽입하여 재조합 발현 벡터를 제작하였다. 상기 전달벡터(pBacPAK8)은 미국 클론텍 회사(CLONTECH Laboratories, Inc. 1020 East Meadow Circle, Palo Alto, CA 94303-4230, USA)에서 구입하여 사용하였다. 상기 재조합 발현 벡터를 제한효소로 처리하여 락토페린 유전자를 확인하였으며, 이를 pBacLf라고 명명하였다. 상기 발현 벡터(pBacLf)의 제조 공정을 도2에 나타내었다.

도3은 선별된 재조합 발현 벡터(pBacLf)의 제한 효소 절단한 후의 전기영동 패턴을 나타낸 사진이다. 레인(lane) 1은 사이즈 마커(size marker)(λ/BstE I)이며, 레인 2는 재조합 발현벡터(pBacLf)의 슈퍼(super) 코일(coil)이

며, 레인 3은 재조합 발현벡터(pBacLf)를 제한효소인 BamH I와 Not I로 처리하여 락토페린 전체 크기 2.1kb에 락토페린 유전자가 절단되어 나타남을 확인한 것이며, 레인 4는 재조합 발현벡터를 제한효소 Eco R V로 처리하여 락토페린 유전자를 확인한 것이며, 레인 5는 재조합 발현벡터(pBacLf)를 제한효소 Sma I로 처리하여 락토페린 유전자를 확인한 것이며, 레인 6는 재조합 발현벡터(pBacLf)를 제한효소 Bgl II로 처리하여 락토페린 유전자를 확인한 것이며, 레인 7은 재조합 발현벡터(pBacLf)를 제한효소 Pst I로 처리하여 락토페린 유전자를 확인한 것이며, 그리고 레인 8은 사이즈 마커를 나타낸 것이다. 도3에 나타난 바와 같이 재조합 발현 벡터를 제한효소인 BamH I과 Not I으로 절단하였을 때 2.1kb에 사람 락토페린 유전자가 나타났다.

실시예 2 : 재조합 곤충세포 Sf-Lf의 선별 및 재조합 바이러스의 동정

Sf9 cell을 10% FBS를 포함하는 그래이스 기초배양기(Grace's basic medium)에 1.0×10^6 세포 정도로 접종하여 4시간 동안 배양한다. 그래이스의 기초배양기로 2번 세척(washing)하여 상온에서 30분간 방치한다. 리포좀-매개(liposome-mediated) 트랜스펙션(transfection) 방법을 이용하기 위해 준비된 재조합 전달 벡터(recombinant transfer vector)와 바이러스 DNA(BacPAK 6, Clontech co.)를 잘 섞어 리포펙틴(lipofectin)과 함께 세포 단층(cell monolayer) 위에 떨어뜨리고 혈청(serum), 항생제(antibiotics)가 포함된 그래이스 배양기에 첨가한 후 28℃에서 5일 동안 배양하였다. 상등액을 배양액으로 3-5회, 10단계로 희석을 하여 직경 60mm 플레이트(plate)에 단층(monolayer)으로 배양된 Sf9 세포에 접종시켰다. 바이러스가 흡착되면 용해시킨 아가로스(agarose) 함유배지를 세포 위에 굳힌다. 6-7일 후, 뉴추럴 레드(neutral red) 용액으로 염색하여 보면, 죽은 세포는 염색되어 구별이 가능하고 동시에 플라크(plaque)를 형성시킨다. 현미경을 사용하여 재조합 바이러스의 감염에 의해 폴리헤드린 단백질이 나타나지 않는 플라크를 선별하여 파스퇴르 피펫(pasteur pipet)으로 아가로스와 함께 빨아 올려 1㎖의 배지 안에 부유시킨다. 이후 재조합 바이러스가 락토페린 유전자를 포함하고 있는지 증명하기 위해 새로운 그래이스 배양기에서 배양된 Sf 9세포에 재조합 바이러스를 또다시 감염시켜 재조합 바이러스 DNA를 분리(isolation)한 후 아가로스 겔(gel) 전기영동(electrophoresis)으로 DNA의 영동 패턴(pattern)을 비교하였으며, 락토

페린을 증폭할 수 있는 프라이머(primer)를 이용하여 PCR(polymerase chain reaction)법으로 확인하였고, 제한효소 처리 후 써던 블랏(southern blot)을 통해 2.1kb 락토페린 유전자를 확인하였다.

도4는 재조합 곤충세포(Sf-Lf)로부터 분리된 재조합 바이러스 DNA로부터 2.1kb의 락토페린 cDNA가 클로닝되어 있음을 보여준다. 레인(Lane) 1은 사이즈 마커(size marker)(λ/BstE II)이며, 레인 2는 음성조절(negative control)(pBacPAK8)이며, 레인 3과 4는 재조합 바이러스(viral) DNA를 나타내는 것이다. 레인(Lane) 3에서 나타나듯이 재조합 바이러스) DNA로부터 사람 락토페린(hLf)만 특이적으로 증폭하는 프라이머를 이용하는 2.1kb의 사람 락토페린 cDNA가 증폭되었음을 보여준다.

도5는 도4에서 확인된 사실을 기초로 하여, 재조합 바이러스 DNA를 제한효소 *BamH* I, *Not* I, *Acc* I 등으로 처리한 후, 써던 블랏(Southern blot)으로 2.1kb의 락토페린 cDNA를 확인 한 것이다. 레인 1은 재조합 바이러스 인텍트(intact) DNA이며, 레인 2는 재조합 바이러스 DNA(BamH I/Not I), 레인 3은 재조합 바이러스 DNA(Acc I)이며, 레인 4는 재조합 바이러스 DNA(BamH I/Not I/Bgl II)이며, 레인 5는 바이러스 DNA로부터 생성된 증폭된 PCR 락토페린 유전자이며, 레인 6은 DIG-Labeled 사이즈 마커이며, 레인 7은 재조합 플라스미드(pBacPAK8) 슈퍼코일(supercoil) DNA이며, 그리고 레인 8은 음성조절(pGEMLf)(BamH I/Not I)을 나타내는 것이다.

음성 대조부(negative control)로서 pBacPAK8을 사용하였으며, 양성 대조부(positive control)로는 2.1kb 락토페린 유전자가 포함된 pGEMLf를 제한효소 *BamH I/Not* I으로 절단하여 사용하였다. 프로브(probe)로는 락토페린 cDNA의 N-lobe의 일부분을 DIG-label하여 사용하였으며, 도5에 나타난 바와 같이 재조합 바큐로바이러스 DNA에서 양성 대조부와 동일한 위치 2.1kb에서 띠(band)가 나타남을 알 수 있다. 이로서 본 발명에서 선별한 재조합 바이러스는 사람 락토페린 DNA를 가지고 있는 것이 증명되었다.

실시예 3 : 재조합 곤충세포(Sf-Lf)로부터 사람 락토페린의 형질발현 및 확인

단백질을 발현하는 것을 확인하기 위하여 재조합 세포를 셀 라이시스 버퍼(cell lysis buffer)(50mM Tris-HCl, pH8.0, 5% 2-머캅토에탄올, 0.4% w/v SDS, 10mM EDTA)로 파쇄하여 쿰아시-블루 폴리아크릴아미드 겔 런닝

(coomassie-blue polyacrylamide gel running)을 수행하였고 안티-락토페린(anti-Lf)을 이용하여 웨스턴 블랏(western blot)을 수행하였다.

도6에 나타난 바와 같이, 재조합 바이러스 저장물(viral stock)을 곤충세포(Sf9)에 감염시켜 4일째 모아 숙주로 사용된 곤충세포(Sf9)와 함께 세포(cell)를 파쇄하여 상층액을 취하여 SDS-PAGE와 안티-락토페린(anti-Lf) Ab로 웨스턴 블랏(western blot)을 수행한 결과 양성 대조부(positive control)로 사용된 초유와 정제된 락토페린 단백질에서 발색되는 80kDa 위치와 동일한 위치에서 띠(band)가 진하게 발색되었다. 상기와 같은 사실로 볼 때, 재조합 곤충세포(Sf-Lf)에서 사람 락토페린 단백질이 생성됨을 알 수 있다. 레인 1은 프로테인 사이즈 마커이며, 레인 2는 초유상등액(Colostral soup)이며, 레인 3은 곤충세포(Sf9)이며, 그리고 레인 4, 5는 재조합 곤충세포를 나타내는 것이다. 발현된 락토페린을 밀도측정법(Densitometry)에 의하여 정량하였을 때 800㎎/ℓ 이상 생산하는 것으로 나타났다. 이와 같은 발현양은 앞서 밝힌 아스페르길러스(Aspergillus) 니듈란스(Nidulans)나 아스페르길러스(Aspergillus) 오리제(Oryzae)의 발현된 락토페린 양에 비해 월등히 많은 양이며, 경제적으로도 생산성이 대단히 높은 것이다.

실시예 4 : 재조합 락토페린의 항균 작용 검증

병원성 미생물에 대한 재조합 곤충세포(Sf-Lf)의 항균작용을 측정하기 위해 상기 곤충세포(Sf-Lf)를 냉동해동(freeze and thaw)방법으로 세포를 파쇄하여 상층액을 취하여 병원성 미생물에 250㎍/㎖정도의 락토페린 농도로 섞어준 후 0분, 15분, 30분, 45분, 60분 간격으로 플레이트 카운트 아가 플레이트(plate count agar plate)에 도말하였다.

표1에 나타낸 바와 같이, 락토페린이 포함된 상등액 만으로도 1시간 안에 병원성 미생물이 사멸되는 것을 세포(cell)의 수를 측정하여 알 수 있었다. 음성 대조부(negative control)로는 Sf 세포를 파쇄하여 재조합 Sf-Lf와 같은 방법으로 병원성 미생물에 미세분석(microassay)를 수행하였으나, 세포의 수가 감소되는 현상을 볼 수 없었다.

상기의 결과로부터, 재조합 곤충세포(Sf-Lf)에서 만들어지는 락토페린 단백질이 항균성을 지니고 있음을 확인 할 수 있었다.

【표 1】

균주	세포수(cfu/mℓ)									
	*E. coli 300		*S. typhimurium 114		@P. putida		@P. fluorescence		@P. cepasia 9613	
	-	+	-	+	-	+	-	+	-	+
0 분	$>10^7$	$>10^7$	$>10^7$	$>10^7$	$>10^7$	6.7×10^6	$>10^7$	1.8×10^6	5×10^5	1.4×10^5
15 분	$>10^7$	$>10^6$	$>10^7$	5.0×10^4	$>10^7$	3.0×10^4	$>10^7$	1.4×10^4	5×10^5	9.8×10^2
30 분	$>10^7$	1.3×10^4	$>10^7$	1.2×10^4	$>10^7$	6.9×10^5	$>10^7$	4.0×10^5	5×10^5	2.1×10^2
45 분	$>10^7$	6.2×10^5	$>10^7$	2.0×10^5	$>10^7$	3.0×10^5	$>10^7$	2.9×10^5	5×10^5	10
60 분	$>10^7$	10^2	$>10^7$	1.2×10^2	$>10^7$	10^2	$>10^7$	5×10^2	5×10^5	0

상기 표1에서 * 는 동물성 병원균을 나타낸 것이며, @ 는 식품오염세균을 나타낸 것이고, + 는 락토페린과 함께 배양한 것을 나타낸 것이며, - 는 락토페린 없이 배양한 것을 나타낸 것이다.

【산업상 이용가능성】

본 발명의 곤충세포를 이용한 사람 락토페린 제조방법에 따라 제조된 재조합 곤충세포(Sf-Lf)는 현탁배양이 가능하여 플라스크 배양이 가능하며, 일반적인 고등 생물세포와는 달리 이산화탄소(CO_2)가 필요 없고, 배양시 FBS(Fetal Bovine Serum)를 사용하지 않아도 되므로 사람 락토페린을 적은 비용으로 손쉽게 대량 생산할 수 있는 발명의 효과를 갖는다.

【청구의 범위】

【청구항 1】

전달 벡터(tansfer vecter)(1)와 재조합 플라스미드(phLf-8)(2)를 조합하여 락토페린 유전자가 벡터(pBacPAK) 내의 폴리헤드린(polyhedrin) 프로모터(promotor)의 조절을 받도록 변형한 재조합 발현 벡터(pBacLf)(3)를 제조하고;

상기 재조합 발현 벡터를 헬프 벡터(pBacPAK6)(4)와 함께 배양기내의 곤충세포(Sf9)(5)에 코트랜스펙션(cotransfection)하여 재조합 곤충세포(Sf-Lf)(6)를 제조하고 상기 재조합 곤충세포로부터 재조합 곤충 바이러스를 제조하고; 그리고

상기 재조합 곤충세포(Sf-Lf)(6)로부터 사람 락토페린을 생산하는 단계;

로 이루어지는 것을 특징으로 하는 곤충세포를 이용한 사람 락토페린의 제조방법.

【청구항 2】

제1항에서, 상기 재조합 곤충세포 제조단계에서 재조합 곤충세포가 배양된 배양액을 원심분리하여 상등액에 존재하는 상기 곤충세포로부터 나온 자손 바이러스(progeny virus)를 얻는 단계를 더 포함하는 것을 특징으로 하는 곤충세포를 이용한 사람 락토페린의 제조방법.

【청구항 3】

전달 벡터(tansfer vecter)(1)와 재조합 플라스미드(2)를 조합하여 락토페린 유전자가 벡터(pBacPAK) 내의 폴리헤드린 프로모터의 조절을 받도록 변형한 재조합 발현 벡터(pBacLf)(3)를 제조하고;

상기 재조합 발현 벡터를 헬프 벡터(pBacPAK6)(4)와 함께 배양기내의 곤충세포(Sf9)(5)에 코트랜스펙션(cotransfection)하여 재조합 곤충세포(Sf-Lf)(6)를 제조하고 상기 재조합 곤충세포로부터 재조합 곤충 바이러스를 제조하고; 그리고

상기 재조합 곤충세포(Sf-Lf)(6)로부터 사람 락토페린을 생산하는 단계;

로 이루어지는 방법으로 제조되는 것을 특징으로 하는 사람 락토페린.

【청구항 4】

전달 벡터(tansfer vecter)(1)와 재조합 플라스미드(2)를 조합하여 락토페린 유전자가 벡터(pBacPAK) 내의 폴리헤드린 프로모터의 조절을 받도록 변형한 재조

합 발현 벡터(pBacLf)(3)를 제조하고;

상기 재조합 발현 벡터를 헬프 벡터(pBacPAK6)(4)와 함께 배양기내의 곤충세포(Sf9)(5)에 코트랜스펙션(cotransfection)한 다음 재조합 곤충세포(Sf-Lf)(6)를 제조하여 배양하고; 그리고

상기 재조합 곤충세포(Sf-Lf)로부터 재조합 곤충 바이러스를 생산하는 단계;

로 이루지는 방법으로 제조되는 것을 특징으로 하는 재조합 곤충 바이러스.

【청구항 5】

제1항의 방법에 의하여 제조된 사람 락토페린을 병원성 미생물과 혼합하여 항균작용을 측정하는 것을 특징으로 하는 재조합 사람 락토페린의 생물학적 검증방법.

【청구항 6】

제5항에서, 상기 병원성 미생물은 슈도모나스 세파시아(*Pseudomonas cepacia*), 슈도모나스 푸티다(*Pseudomonas putida*), 살모넬라 티피뮤리움(*Salmonella typhimurium*), 슈도모나스 플루오레슨스(*Pseudomonas fluorescence*) 및 이 콜라이(*E. coli*)로 이루어지는 군으로부터 선택되는 것을 특징으로 하는 사람 락토페린의 생물학적 검증방법.

【요약서】

【요약】

본 발명은 곤충세포를 이용하여 제조된 사람 락토페린(Lactoferrin) 및 그 제조방법에 관한 것으로, 상기 제조방법은 전달 벡터(tansfer vecter)(1)와 재조합 플라스미드(2)를 조합하여 락토페린 유전자가 벡터(pBacPAK) 내의 폴리헤드린(polyhedrin) 프로모터(promotor)의 조절을 받도록 변형한 재조합 발현 벡터(pBacLf)(3)를 제조하고; 상기 재조합 발현 벡터를 헬프 벡터(pBacPAK6)(4)와 함께 배양기내의 곤충세포(Sf9)(5)에 코트랜스펙션(cotransfection)하여 재조합 곤충세포(Sf-Lf)(6)를 제조하고 상기 재조합 곤충세포로부터 재조합 곤충 바이러스를 제조하고; 그리고 상기 재조합 곤충세포(6)로부터 사람 락토페린을 생산하는 단계로 이루어진다.

본 발명의 재조합 사람 락토페린의 생물학적 검증방법은 재조합 사람 락토페린을 재조합 곤충세포로부터 추출하여 슈도모나스 세파시아(*Pseudomonas cepacia*), 슈도모나스 푸티다(*Pseudomonas putida*), 슈도모나스 플루오레슨스(*Pseudomonas fluorescence*), 살모넬라 티피뮤리움(*Salmonella typhimurium*), 이 콜라이(*E. coli*)와 같은 병원성 박테리아와 혼합하여 병원성균의 사멸 정도를 측정하는 것이다.

【대표도】

도 1